Comprehensive biometric, biochemical and histopathological assessment of nutrient deficiencies in gilthead sea bream fed semi-purified diets

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Abstract

Seven isoproteic and isolipidic semi-purified diets were formulated to assess specific nutrient deficiencies in sulphur amino acids (SAA), long-chain polyunsaturated fatty acids (n3 LC-PUFA), phospholipids (PL), phosphorus (P), minerals (Min) and vitamins (Vit). The control diet (CTRL) contained these essential nutrients in adequate amounts. Each diet was allocated to triplicate groups of juvenile gilthead sea bream fed to satiety over an 11-week feeding trial. Weight gain of n/3 LC/PUFA, P-Vit, and PL-Min-SAA groups was 50%, 60-75% and 80-85% of CTRL group, respectively. Fat retention was decreased by all nutrient deficiencies except by Min diet. Strong effects on N retention were found in n3 LC-PUFA and P fish. Combined anaemia and increased blood respiratory burst were observed in n3 LC-PUFA fish. Hypoproteinemia was found in SAA, n3 LC-PUFA, PL and Vit fish. Derangements of lipid metabolism were also a common disorder, but the lipodystrophic phenotype of P fish was different to that of other groups. Changes in plasma levels of electrolytes (calcium, phosphate), metabolites (creatinine, choline) and enzyme activities (alkaline phosphatase) were related to specific nutrient deficiencies in PL, P, Min or Vit, whereas changes in circulating levels of growth hormone and insulin-like growth factor-I primarily reflected the intensity of the nutritional stressor. Histopathological scoring of liver and intestine segments showed specific nutrient-mediated changes in lipid cell vacuolisation, inflammation of intestinal submucosa, and the distribution and number of intestinal goblet and rodlet cells. These results contribute to define the normal range of variation for selected biometric, biochemical, haematological and histochemical markers.

1. Introduction

Clinical haematology and basic blood biochemistry are common diagnostic tools to assess health and welfare in humans and most livestock production systems[1,2]. In fish, although there is experimental evidence that circulating electrolytes, metabolites and hormones highly reflect impaired growth performance, stress condition and disease outcome, the use of such analyses as diagnostic tools is poorly established in practice. This is due to the paucity of reliable information on reference values of haematology and blood biochemistry parameters in healthy and well-nourished fish. Some attempts have been made to compile available data in fish blood biochemistry and haematology[3-6]. However, for the majority of well-established farmed finfish, grown under different rearing conditions with diverse physiological status, validated data are lacking. Besides, important gaps on reliable clinical biomarkers are arising with the advent of new fish feeds with a maximized replacement of fish meal (FM) and fish oil (FO) by alternative feedstuffs of terrestrial or marine origin. Similarly, for the histopathological scoring of relevant target tissues (liver, intestine), there is evidence of clinical signs of liver steatosis, accumulation of intestinal lipid droplets or intestine submucosa inflammation arising from lipid-related metabolic disorders[7,8], but a direct link to a specific nutrient or a group of nutrients is lacking.

Therefore, there is an urgent need for reliable reference values, but also for the definition of blood and histopathological parameters that have specificity, sensitivity and diagnostic value for nutritional deficiencies. Thus, our experimental setup with a feeding trial of semi-purified diets formulated for a given nutritional deficiency in a typically marine fish, such as gilthead sea bream, considered two major steps: i) functional validation of a set of clinical data based on body composition, organosomatic indices, and blood haematology and biochemistry for the initial assessment of nutrient deficiencies in methionine (Met), essential fatty acids (EFA) such as the n-3 long-chain polyunsaturated fatty acids, phospholipids (PL), phosphorous (P) and micronutrients (minerals, vitamins) and ii) histopathological scoring of liver and intestine sections as a complementary diagnostic tool. The studied nutrient deficiencies were chosen because they are constraining factors in practical marine fish feeds with a maximized FM/FO replacement. In parallel, current work is underway for the definition of the normal range of variation of selected biomarkers, integrating the data reported herein with our own data in the framework of the ARRAINA (Advanced Research Initiatives for Nutrition

and Aquaculture) EU project, where fish were fed through the production cycle with varying inclusion levels of FM and FO (from 40% in control diet to 7.5% in the extreme low FM/FO diet).

2. Experimental methods

2.1. Diets

Seven isonitrogenous (51-52% of dry matter, DM) and isolipidic (14.5-15.5% of DM) diets were formulated. They were produced in a semi-industrial scale (Sparos LDA, Algarve, Portugal) (Table 1). All diets contained casein (20%), casein hydrolysate (5%), gelatin (5.8%) and soy protein concentrate (34.5%) as protein sources, and were supplemented with L-threonine (0.02%). Taurine (0.3%), betaine (0.3%) and glucosamine (0.4%) were added as attractants, and ethoxyquin (0.1%) as antioxidant in all diets. DL-Methionine was supplemented at 0.4% in all diets except the diet designed to be deficient in sulphur amino acids (SAA diet). FO was added at the 13.9% in all diets except in the fatty acid (FA) deficient diet (n-3 LC-PUFA diet), in which FO was totally replaced by a blend of vegetable oils (VO) in order to reduce the EPA (eicosapentaenoic acid) and DHA (docosapentaenoic acid) contents to trace levels (Table 2). Soy lecithin (2%) was added as the unique source of PL in all diets except in the PL deficient diet. Calcium phosphate (2.2%) was added in all diets except in the P deficient diet. Mineral premix based on available data on mineral requirements of fish[(9)] was included at 2.2% in all diets except in the diet designed to be mineral deficient (Min diet). Vitamin premix based on NRC[(10)] was added in all diets at an incorportaion level of 2% except in the diet designed to be vitamin deficient (Vit diet).

2.2. Feeding trial and fish sampling

Juvenile sea bream of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were acclimatised to laboratory conditions for one month before the start of a 12 weeks trial (May-July) in the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS-CSIC). Following the acclimatisation period, fish of 15 g initial mean body weight were randomly distributed into 500 L-tanks in triplicate

groups of 35 fish each. Fish were fed to visual satiety one (12 h)/two times (9h, 14 h) per day (6 days per week). The trial was conducted under natural photoperiod and temperature conditions at IATS latitude (40°5N; 0°10E), increasing water temperature from 19 ºC in May to 24ºC at the end of July. Water flow rate was 20 L/min, oxygen content of water effluents was always higher than 85% saturation, and unionised ammonia remained below toxic levels (<0.02 mg/L).

At the end of the trial and following overnight fasting (10-12 h in the morning), 18 fish per dietary treatment (six per tank) were randomly selected and decapitated under anaesthesia with 3-aminobenzoic acid ethyl ester (MS-222, 100 μg/mL). Blood was taken from caudal vessels with heparinised syringes (less than five minutes for all the fish sampled from each tank). One aliquot was used for measurements of haematological parameters and respiratory burst activity of blood leukocytes. The remaining blood was centrifuged at 3,000 g for 20 min at 4ºC, and the resulting plasma was stored in separate aliquots at -20ºC until further assays were performed. Viscera, liver and mesenteric fat were weighed and representative portions of liver and intestine segments (anterior and posterior) were taken for histological processing. When blood and tissue collection was completed, additional fish (4 fish per tank) were taken for whole body composition analyses.

The experimental protocol was approved by the Agencia Estatal Consejo Superior de Investigaciones Científicas, IATS Review Board, and all procedures were in accordance with national and current EU legislations on the handling of experimental animals.

2.3. Chemical composition

Diets and fish for body composition analyses (a pooled sample of 10 fish at the beginning and pools of 4 fish per tank at the end of trial) were ground, and small aliquots were dried to determine moisture content. The remaining samples were freeze-dried and chemical analyses were made: dry matter by drying at 105 °C for 24 h, protein (N x 6.25) by the Kjeldahl method, and fat after dichloromethane extraction by the Soxhlet method.

Total lipids for analyses of dietary FA acid composition were extracted by the method of Folch et al.[(11)], with chloroform-methanol (2:1, v v-1) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. After the addition of nonadecanoic FA

(19:0) as internal standard, total lipids were subjected to acid-catalysed transmethylation for 16 h at 50°C using 1 mL toluene and 2 mL of 1% (v v-1) sulphuric acid in methanol[12]. FA methyl esters (FAME) were extracted with hexane:diethyl ether (1:1) and purified by thin layer chromatography (Silica gel G 60, 20 × 20 cm glass plates, Merck, Darmstadt, Germany), using hexane:diethyl-ether:acetic acid (85:15:1.5) as a solvent system. FAMEs were then analysed with a Fisons Instruments GC 8000 Series (Rodano, Italy) gas chromatograph as described elsewhere[13]. Individual FAMEs were identified by comparison with well characterised sardine oil (Marinol, Fishing Industry Research Institute, Rosebank, South Africa) and the FAME 37 mix from Supelco (Bellefonte, PA, USA). BHT and internal standards (19:0) were obtained from Sigma-Aldrich (Madrid, Spain). All solvents in lipid extraction and FA analyses were HPLC grade and were obtained from Merck.

2.4. Haematology

Haemoglobin (Hb) concentration was determined with a HemoCue B-Haemoglobin Analyser® (AB, Leo Diagnostic, Sweden), which uses a modified azide methaemoglobin reaction for Hb quantification. The haematocrit (Hct) was measured after centrifugation of the blood in heparinised capillary tubes at 13,000 g for 10 minutes. Red blood cell (RBC) counts were made in a Neubauer chamber using an isotonic solution (1% NaCl). Erythrocyte osmotic fragility test was made in hypotonic NaCl solutions with haemolysis read at 540 nm. Median corpuscular fragility (MCF) was defined as the concentration of NaCl (g/L) causing 50% lysis.

2.5. Blood biochemistry

Plasma glucose was measured by the glucose oxidase method (ThermoFisher Scientific, Waltham, Massachusetts, USA). Plasma triglycerides (TG) were determined using lipase/glycerol kinase/glycerol-3phosphate oxidase reagent. Total plasma cholesterol was determined using cholesterol esterase/cholesterol dehydrogenase reagent (ThermoFisher Scientific). HDL and LDL/VLDL cholesterol were determined with the kit (EHDL-100) of BioAssay Systems (Hayward, California, USA), based on an improved polyethylene glycol precipitation method in which HDL and LDL/VLDL are separated, and cholesterol concentrations are determined using cholesterol

esterase/cholesterol dehydrogenase reagent. Total plasma proteins were measured with the Bio-Rad protein reagent (Hercules, California, USA) with bovine serum albumin as standard.

Changes in plasma enzyme activities of alanine aminotransferase (ALAT, EC 2.6.1.2) (EALT-100), aspartate aminotransferase (ASAT, EC 2.6.1.1) (EASTR-100) and glutamate dehydrogenase (GLDH, EC 1.4.1.2) (DGLDH-100) were measured using colorimetric assay kits (BioAssays Systems). Plasma alkaline phosphatase (ALP, EC. 3.1.3.1) activity was determined by a fluorimetric assay kit (QFAP-100, BioAssays Systems).

Plasma levels of creatinine (DICT-500), choline (ECHO-100), calcium (DICA-500), chloride (DICL-250), magnesium (DMG-250) and phosphate (DIPI-500) were measured by colorimetric assay kits (BioAssays Systems). Total antioxidant capacity was measured as Trolox activity using a microplate assay kit (709001) (Cayman Chemical, Ann Arbor, Michigan, USA). Plasma lysozyme activity was measured by a turbidimetric assay[14] adapted to microplates, as previously described[15]. Induction of the respiratory burst (RB) activity in blood leukocytes was measured directly from heparinised blood, following the method described by Nikoskelainen et al.[16] with some modifications[17].

Plasma GH was determined by a homologous gilthead sea bream radioimmunoassay (RIA) as reported elsewhere[18]. The sensitivity and midrange (ED50) of the assay were 0.15 and 1.8 ng/mL, respectively. Plasma IGFs were extracted by acid-ethanol cryoprecipitation[19], and the concentration of IGF-I was measured by means of a generic fish IGF-I RIA validated for Mediterranean perciform fish[20]. The sensitivity and midrange of the assay were 0.05 and 0.7–0.8 ng/mL, respectively.

2.6. Histology

For histological examination, pieces of liver, anterior (a piece inmediately after the piloric caeca) and posterior (a piece immediately before the rectum) intestinal segments were fixed in 10% buffered formalin, embedded in paraffin, 4 µm-sectioned and stained with Giemsa and periodic acid-Schiff (PAS). Histochemical reactivity in tissues was evaluated by grading staining using the following scale: negative (−), slight (+), moderate (++), and marked (+++).

2.7. Statistical analysis

Data on growth performance and blood haematology and biochemistry were analysed by one-way ANOVA followed by Student Newman-Keuls (SNK) test ($P < 0.05$). All analyses were performed with the SPSS 17.0 program (SPSS, Inc. Chicago, IL, USA).

3. Results

3.1. Growth performance

Data on growth, somatic indexes and body composition are shown in Table 3. As a general rule, nutrient deficient diets reduced significantly feed intake, growth rates and feed efficiency in fish fed P and n-3 LC-PUFA diets. This resulted in a weight gain of 50% (n/3 LC/PUFA), 60-75% (P, Vit) and 80-85% (PL, Min, SAA) of CTRL fish.

Mesenteric fat index (MSI) was markedly reduced in Vit fish. The same was observed in PL and n-3 LC-PUFA groups, although there were no statistically significant differences. Conversely, MSI was significantly increased in P fish. Hepatosomatic index (HIS) was also altered by dietary treatments, and it was largely increased in n-3 LC-PUFA fish. The opposite was found in Min fish and in a lower extent in Met and Vit fish.

Dietary treatment also altered whole body composition with a low protein and lipid content in fish fed P and Vit diets, respectively. This feature was related to a strong decrease in N retention in P and n-3 LC-PUFA fish. Lipid retention was significantly reduced by nutrient deficiencies in all experimental groups with the exception of Min fish.

3.2. Blood analyses

Data on blood analysis are shown in Table 4. Hb concentration, Hct and RBC counts were significantly lower in n-3 LC-PUFA fish than in CTRL fish. This feature was related to a greater osmotic fragility, evidenced by the significant increase of MCF values from 6.6 g/L in CTRL fish to 7.4 g/L in n-3 LC-PUFA fish. Other experimental

groups did not show any statistically significant alteration of haematological parameters.

Blood biochemistry was altered in a nutrient specific manner, and a strong hypotriglyceridemia, hypocholesterolemia and hypoproteinemia with decreased plasma levels of creatinine were found in n-3 LC-PUFA fish, but also in Vit fish. Hypoproteinemia was a sign of SAA and PL deficiency, whereas hypertriglyceridemia and hypercholesterolemia were characteristic features of P fish. Low plasma choline levels were found in SAA, Min and Vit fish.

Plasma electrolytes were highly refractory to dietary treatment in our experimental conditions with the exception of calcium and phosphate in fish fed diets non-supplemented with the vitamin premix and inorganic P, respectively. Likewise, enzyme activities of ALAT, ASAT and GLGH were not modified by dietary treatment, whereas ALP activity was significantly decreased in PL fish but increased in P and Min fish groups. Lysozyme activity was not altered by any dietary treatment. By contrast, RB was triggered in a consistent manner in n-3 LC-PUFA fish. Plasma antioxidant capacity was also increased by nutrient deficiencies, although this feature was especially evident in fish fed P, Min and Vit diets.

Regarding growth factors, circulating levels of GH highly reflected the impairment of growth performance and the highest plasma concentration was observed in n-3 LC-PUFA fish, followed by fish fed P and Vit diets. The opposite was found for circulating levels of IGF-I and the lowest IGF-I concentration was found in n-3 LC-PUFA and P fish groups.

3.3. Histopathological traits

The histological examination of liver and intestine showed different features, which are summarised in Table 5. Representative differential microphotographs are also provided in Figures 1 to 3. The highest level of fat accumulation either in the liver or anterior intestine was observed in fish fed n-3 LC-PUFA, but without reaching steatosis. Accumulation of glycogen in the liver (revealed by PAS staining) was observed in fish fed SAA, P and Vit diets, but it was not extreme. No fat accumulation was observed in the posterior intestine in any of the groups. Goblet cell content and number varied with the diet, a clear decrease in the number of neutral mucins (stained with PAS) was observed at anterior intestine of fish fed n-3 LC-PUFA, PL, P and Min diets. In all fish

groups the number of PAS+ goblet cells was lower at posterior intestine than at anterior intestine, and only PL and Vit fish had slight staining. The number of Giemsa-stained goblet cells was also decreased in P, Min and Vit fish. The staining of the epithelial layer of the posterior intestine was biphasic in the samples from all the experimental diets, except SAA and PL, in which it was homogenous as in CTRL ones. The number of granulocytes in the submucosae at anterior intestine and posterior intestine was not outstanding, except in Vit at anterior intestine. Vit was the only diet in which intraepithelial lymphocytes were in higher number than in the CTRL diets in both intestinal segments. It was also remarkable the high number of rodlet cells in the posterior intestine epithelium of PL, P, Min and Vit fish, with the highest level in Min fish.

4. Discussion

Comprehensive approaches have been used to address the total or partial FM/FO replacement in a wide range of finfish, including rainbow trout[21] and typically marine fish, such as European sea bass[22] and gilthead sea bream. In particular, for gilthead sea bream, the long-term consequences of feeding low FM/FO feeds on growth performance and endocrine status[7,23,24], health and welfare [17,25-30], fish quality[13,31,32] and food safety[33,34] have been considered in a highly integrated manner. However, knowledge on the specific effects and consequences of a given nutrient or a group of nutrients is mostly lacking. Thus, this is one of the first studies analysing at the same time the effects of dietary deletion or reduction of six different nutrients recognised as essential to fish[10], which contributes to fill the gaps in the diagnosis of the nutritional fish condition under standardised rearing conditions. Of note, we did not have the same degree of deficiency for the six different nutrients tested. Indeed, EPA and DHA contents were reduced to trace levels in the n-3 LC-PUFA diet with the total replacement of FO by VO. Likewise, the main source of P in CTRL diet was the added calcium phosphate, but more difficult was to induce a severe Met deficiency preserving the supply of other essential amino acids.

Adipose tissue (AT) is now recognised as an important target tissue for the diagnosis and treatment of most lipid metabolic disorders arising from an excessive lipid influx[35]. Clinically, lipotoxicity not only appears with fattening, but also with hypoxia, blockage of glucocorticoid sensitive pathways, and the acquisition and

maintenance of inflammatory phenotypes[36-38]. In humans as well as in other animal models, FO and n-3 LC-PUFA of FO are able to reverse these clinical symptoms, decreasing lipolysis and alleviating the inflammatory condition of AT, which, in turn, reduces the production of lipolytic cytokines, the release of free FA and thereby the risk of hepatic steatosis[39]. This liver syndrome is the result of a massive synthesis and/or deposition of TG in the form of lipid vacuoles, and it is commonly observed in many fish species challenged with xenobiotics and unbalanced diets[40]. This metabolic derangement is often accompanied by the displacement of the nucleus of hepatocytes and even pyknosis. Relatively little is known on the ultimate mechanism, although the dietary protein source and protein/energy ratio have a major effect on the regulation of lipid metabolism in European sea bass[41,42]. The replacement of FM and FO with plant protein and oil sources also has a number of effects on the regulation of intermediary metabolism in trout[43] and Atlantic salmon[44]. In gilthead sea bream, tissue FA uptake[45,46] and mitochondrial respiration uncoupling[47] are highly affected by FM and FO replacement with plant ingredients. In addition, increases in cell size and lipolytic rates are characteristic features of isolated adipocytes from fish fed either plant proteins or VO[48,49], and lipoid liver degeneration is frequently observed as a metabolic disturbance in gilthead sea bream fed high plant ingredient-based feeds[7,23,50]. Accordingly, the present study shows that the total replacement of FO by VO in FM-free diets (n-3 LC-PUFA diet) caused a slight reduction of MSI related to a lipodystrophic phenotype with clinical signs of hypolipidemia and hepatomegaly (high HSI). The loss of mesenteric AT mass, together with low plasma levels of choline and calcium, was even higher in the reduce vitamin supply. However, abnormal liver lipid deposition rates was not found by light microscopy in this group of fish. Therefore, the absence or modification of AT mass cannot be used as the only criterion for the risk assessment of liver steatosis.

The main signs of P deficiency in fish nutritional studies are poor bone mineralisation and bad growth performance[9,51], which were correlated in the present study with low plasma phosphate concentrations and high plasma activities of ALP in the absence of apparent skeletal deformities. ALP activity is a well-known bone turnover biomarker [52], but herein we also observed an increased plasma ALP activity with the reduced dietary supply of trace minerals (iron, magnesium, zinc, copper and selenium) in Min fish. However, the increase in MSI in combination with hyperlipemia allows differentiating the effects of deficiency in P from those due to deficiencies in

trace minerals. In this sense, it must be noted than an important role of P in the regulation of lipid metabolism has also been reported in other fish species[53,54].

Hct and Hb values are general indicators of health, and these haematological parameters change in response to nutrient deficiencies, environmental conditions, growth status and anti-nutritional factors[55,56]. A high incidence of anaemia has been reported in yellowtail and parrot fish fed FM-free diets[57,58]. This has been attributed, at least in part, to taurine deficiency, and thus its supplementation seems to be required when these fish are fed low levels of taurine in FM or plant protein-based diets[59]. In our study, all the diets were supplemented with taurine (0.3%), and anaemia signs, such as low RBC, Hct and Hb values were found only in fish fed the n-3 LC-PUFA diet, which also showed an increased erythrocyte osmotic fragility, as reported in rats with deficiencies in LC-PUFA[60]. Importantly, this clinical sign was not found in the PL fish group, and therefore contributes to better define the sometimes-overlapping signs of nutrient deficiencies in EFA and PL.

Previous studies in gilthead sea bream have already highlighted increased RB of blood leukocytes in fish fed diets with a high replacement of FO by VO[17,25]. This is confirmed herein and importantly RB turned out to be a specific criterion for the diagnosis of n-3 LC-PUFA deficiency, as it was notably increased in this group and not with PL or other nutrient-induced deficiencies. Likewise, a close talk between PL and bone metabolism exists and the decrease in plasma ALP activity is becoming an easy and highly valuable marker of PL deficiencies in gilthead sea bream. Of note, a high PL supply is required during early life fish stages to improve survival rates and to decrease the incidence of skeletal deformities[61], but PL requirements in juvenile and adult fish are still controversial[62,63].

Plasma transaminases and GLDH are commonly used in clinical chemistry as markers of tissue damage[64], but the current results indicate that they are poorly informative of nutritionally-mediated metabolic derangements in gilthead sea bream. By contrast, overall plasma total antioxidant activity was increased in parallel to the reduced growth performance, indicative of a reduced aerobic metabolism and, therefore, of a reduced production of reactive oxygen species (ROS). Strong support for this comes from inbreeding selection of rat strains, which demonstrates that most stressful and oxidative risk factors correlate with the low expression of genes required for mitochondrial biogenesis and oxidative phosphorylation[65]. Experimental evidence also indicates that plasma antioxidant capacity is increased by hypoxia exposure, probably

due to the concurrent decrease of basal metabolism, mitochondrial respiration uncoupling and oxidative phosphorylation[66]. However, the magnitude and even the direction of the change is poorly predictable when comparisons are made between this and previous feeding trials with practical diets containing FM[17].

Most growth regulatory events of fish are mediated at hormonal level by the GH/IGF axis, keeping pituitary GH secretion and hepatic/extra-hepatic IGF production under control[67-69]. Hence, circulating GH and IGF-I are one of the most important endocrine determinants of growth in a vast array of stress and nutritional disorders arising from crowding and handling stress[70], changes in ration size[68,71], dietary protein/energy ratio[72,73] and dietary protein and lipid sources[23,24,74]. This notion is also found herein, and importantly a close positive correlation between growth rates and circulating levels of IGF-I was evidenced regardless of the nutrient deficiency. As expected, an opposite trend was found for GH and growth rates, which would reflect a lowered negative feedback inhibition of IGFs upon pituitary GH release as a result of a transcriptional defect in the signal transduction of GH receptors. This metabolic feature leads to liver GH resistance and reduced hepatic IGF production in spite of increased plasma levels of GH, as it has been early stated in a wide range of fish species, including gilthead sea bream[71,75-77].

Histological traits also gave interesting information on the possible pathological outcome of nutrient deficiencies. The highest accumulation of hepatic lipids was found in gilthead sea bream fed the n-3 LC-PUFA deficient diet, followed by SAA, P and Min. However, lipid accumulation did not reach the highest score of steatosis observed by us with other dietary interventions[7,15], or by other authors using diets with an excess of dietary lipids[78], EFA deficiencies[79] and VO[50,80,81]. Glycogen accumulation was also high in the liver of SAA, P and Vit fish, although the observed glycogen deposition did not reach an extreme condition. Massive accumulation of supranuclear lipid droplets at the intestinal epithelial layer is also considered a sign of inadequate/unbalanced diets due to a reduced metabolisation of absorbed lipids, either because they are not needed or because they are absorbed in a higher amount than needed. This accumulation was not observed at the posterior intestine for any diet, but it was a clear feature at the anterior intestine after feeding with n-3 LC-PUFA, PL and Vit deficient diets. This epithelial accumulation stands as an earlier marker of deregulated lipid metabolism compared to lipid accumulation in the liver, as it was visible in PL and Vit fed fish, in which lipoid liver degeneration was not found.

Another histological feature with clear differences among fish was the number of goblet cells and their staining characteristics. In this sense, it is noteworthy that the n3 LC-PUFA diet induced a strong reduction in the number of goblet cells, and the remaining goblet cells were not stained either by Giemsa or PAS, indicating that mucin content was not neutral or acidic. Neutral mucins were also absent at anterior intestine as a result of the feeding with all the diets except SAA and Vit, and at posterior intestine except PL and Vit. Previous gilthead sea bream studies indicate that other models of nutritional- and parasite-induced enteritis also invoke modifications in goblet cells type and number[26,82]. In fact, the 66% replacement of FO by VO in plant protein-based diets produced a significant decrease in GC with neutral and acidic mucins at anterior intestine and medium intestine and also in those with carboxylic mucins and sialic acid at medium intestine, but no significant changes at posterior intestine. In European sea bass fed with mannan oligosaccharides, the number of goblet cells secreting acidic mucins was increased[83]. In yellow perch fed with wheat-gluten-protein-based diets even supplemented with free lysine, the number of goblet cells was also decreased[84]. By contrast, Atlantic salmon[85], Atlantic cod[86] and carp[87] fed high-plant-protein-based diets presented goblet cells hypertrophy and hyperplasia.

Rodlet cells are exclusive of teleost epithelial layers and represent a cell type whose function has not definitively been established, although considered to be closely linked to the immune system and osmoregulation[88]. Many studies consistently report an association between rodlet cells proliferation/ hyperplasia and the presence of a variety of parasites, chemicals and environmental stressors[89,90] and they even have been proposed as biomarkers[89]. However, there is no previous report on the relationship with the diet. In the current study, the number of rodlet cells was increased only at posterior intestine, notably in fish fed Min diet. The increased presence of Rodlet cells could therefore be interpreted either as a sign of inflammation or osmoregulatory imbalance and reinforce the idea that cell osmoregulation is dependent on trace minerals rather than P uptake. The higher presence of rodlet cells was coincident with other inflammatory markers (intraepithelial lymphocytes and submucosal granulocytes) in fish feed Min, PL and Vit diets either at anterior intestine or posterior intestine. In any case, the observed cellular inflammation was mild and by no means comparable with that caused by other dietary interventions in Atlantic salmon[91], common carp[87] or gilthead sea bream[15].

In summary, clinical blood biochemistry and tissue histopathology have been proved highly informative to assess nutritional condition of farmed gilthead sea bream. The final diagnosis outcome might require confirmation by more specific assays, but the generated information is by itself useful for the overall assessment of fish performance and metabolic condition when the measured parameters are referred to a control group or historical data for a given fish strain, life stage and/or rearing condition. All this information is summarised in Supplemental Table 1 as a set of clinical signs for a given nutritional deficiency in gilthead sea bream. To establish the normal range of variation of these parameters as a function of season and growth performance, the data from this study were combined with those derived from other past and on-going feeding trials with practical diets. The rationale for this is to cover a wide range of variation for marine and plant ingredients without apparent detrimental effects on fish performance through the production cycle. The reference values for these studied biomarkers are shown in Supplemental Table 2, and will be periodically updated on the basis of the data produced within the ARRAINA project.

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Figure legends

Figure 1. Histochemical staining of liver sections from gilthead sea bream fed the control diet (A, B), the CL-PUFA diet (C, D) or the SAA diet (E, F). Stainings: Giemsa (A, C, E), PAS (B, D, F). Scale bars = 20 µm.

Figure 2. Histochemical staining of anterior intestine sections from gilthead sea bream fed the control diet (A, B), the CL-PUFA diet (C, D) or the Vit (E, F). Stainings: Giemsa (A, C, E), PAS (B, D, F). Scale bars = 20 µm.

Figure 3. Histochemical staining of posterior intestine sections from gilthead sea bream fed the control diet (A, B), the CL-PUFA diet (C, D) or the Min (E, F). Stainings: Giemsa (A, C, E), PAS (b, d, f). Scale bars = 20 µm. Inset in C shows detail of rodlet cells in the epithelium; inset in E shows the abundant granulocytes in the submucosa.

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777 the intestines of fasted fish. *J Fish Dis* **19**, 375-387.
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781 **Table 1.** Ingredients and chemical composition of experimental diets.

	Diet						
	CTRL	SAA	n-3 LC-PUFA	PL	P	Min	Vit
Ingredient (%)							
Casein	20	20	20	20	20	20	20
Casein hydrolisate	5	5	5	5	5	5	5
Gelatin	5.78	5.78	5.78	5.78	5.78	5.78	5.78
Soy protein concentrate (Soycomil PC)	34.5	34.5	34.5	34.5	34.5	34.5	34.5
DL-Methionine	0.4	0	0.4	0.4	0.4	0.4	0.4
L-Threonine	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Dextrine	11.2	11.2	11.2	11.2	11.2	11.2	11.2
Soy lecithin	2	2	2	0	2	2	2
Fish oil	13.9	13.9	0	13.9	13.9	13.9	13.9
Vegetable oil	0	0	13.9	0	0	0	0
Cellulose	0	0.4	0	0	2.2	1.5	1.5
CaHPO4.2H2O (18%P)	2.2	2.2	2.2	2.2	0	2.2	2.2
Mineral premix-INRA[1]	2	2	2	2	2	0.5	2
Vitamin premix-INRA[2]	2	2	2	2	2	2	0.5
Taurine	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Betaine	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Glucosamine	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Ethoxyquin (75%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Proximate composition							
Dry matter (DM, %)	92.4	92.5	95.4	93.3	95.5	92.2	92.8
Crude protein (% DM)	51.8	51.5	50.8	51.1	50.8	52.0	52.5
Crude fat (% DM)	15.0	15.1	15.5	15.6	14.4	14.7	14.7
EPA+DHA (% DM)	2.68	2.68	0.03	2.83	2.60	2.64	2.37

782 [1]Supplied the following (g/kg mix): calcium hydrogen phosphate 500, calcium carbonate
783 (40% Ca) 215, sodium chloride 40, ferrous sulphate (21% Fe) 20, manganese sulphate 3,
784 zinc sulphate 4, copper sulphate 3, cobalt (II) chloride (25% Co) 0.02, potassium iodine
785 0.04, sodium selenite 0.03, sodium fluoride 1, magnesium hydroxide (60% Mg) 124,
786 potassium chloride 90.
787 [2]Supplied the following (g/kg mix, except as noted): retinyl acetate 1, DL-cholecalciferol
788 2.5, DL-α tocopheryl acetate 5, menadione sodium bisulphite 1, ascorbic acid 20, thiamin
789 0.1, riboflavin 0.4, pyridoxine 0.3, vitamin B12 10 mg, nicotinic acid 1, pantothenic acid
790 2, folic acid 0.1, biotin 10 mg, choline chloride 200, inositol 30.

791 **Table 2.** Fatty acid composition of experimental diets (% total FAME).

Fatty acid	Diet						
	CTRL	SAA	n-3 LC-PUFA	PL	P	Min	Vit
14:0	6.81	7.06	0.51	7.38	7.03	7.06	7.50
15:0	0.61	0.74	0.24	0.79	0.76	0.68	1.01
16:0	17.56	18.11	15.48	18.53	18.58	18.47	19.13
16:1n-7	7.36	7.44	0.24	7.60	7.29	7.31	7.46
16:2	0.91	0.92	0.12	1.06	1.02	1.23	1.13
16:3	1.25	1.25	0.06	1.29	1.20	1.26	1.21
16:4	1.37	1.37	0.08	1.41	1.31	1.36	1.30
17:0	0.48	0.49	tr	0.52	0.51	0.51	0.53
18:0	3.36	3.47	4.68	3.48	3.62	3.56	3.69
18:1n-9	10.87	10.79	30.57	10.68	10.84	10.76	10.95
18:1n-7	2.49	2.52	0.98	2.57	2.52	2.52	2.57
18:2n-6	3.98	3.87	16.46	2.09	3.81	3.81	3.66
18:3n-3	1.10	1.07	27.44	1.10	1.13	1.09	1.01
18:3n-6	0.30	0.30	0.09	0.31	0.29	0.30	0.29
18:4n-3	2.21	2.22	tr	2.26	2.14	2.23	2.07
20:0	0.29	0.28	0.32	0.29	0.30	0.29	0.33
20:1n-9	2.32	0.81	0.38	2.51	2.46	2.43	2.48
20:1n-7	0.21	0.21	tr	0.22	0.21	0.21	0.22
20:2n-6	0.15	0.15	0.07	0.15	0.15	0.15	0.15
20:3n-3	0.07	0.07	tr	0.07	0.07	0.07	0.06
20:3n-6	0.07	0.05	tr	0.05	0.05	0.05	0.06
20:4n-6	0.90	0.91	tr	0.92	0.88	0.91	0.85
20:4n-3	0.61	0.61	tr	0.62	0.60	0.61	0.58
20:5n-3 (EPA)	12.98	13.01	0.18	13.17	12.56	13.06	11.88
21:0	0.08	0.10	tr	0.08	0.09	0.09	0.08
21:5n-3	0.48	0.49	tr	0.49	0.47	0.49	0.44
22:0	0.12	0.13	0.19	0.11	0.13	0.13	0.14
22:1n-11	3.34	3.58	tr	3.66	3.69	3.61	3.61
22:1n-9	0.20	0.11	0.05	0.11	0.08	0.11	0.10
22:4n-6	0.08	0.08	tr	0.08	0.08	0.09	0.08
22:5n-3	1.39	1.40	0.10	1.41	1.36	1.40	1.27
22:6n-3 (DHA)	9.94	9.95	0.07	10.05	9.60	9.98	8.96
Total	94.14	93.81	98.37	95.28	95.10	95.00	96.08
Saturates	29.31	30.38	21.44	31.19	31.03	30.79	32.40
Monoenes	26.79	25.48	32.26	27.34	27.09	26.96	27.39
n-6 LC-PUFA [1]	0.98	0.99	tr	1.00	0.95	0.99	0.93
n-3 LC-PUFA[1]	25.41	25.45	0.38	25.73	24.59	25.55	23.13

792 Values are means of two determinations; tr = trace value < 0.05.
793 [1] Fatty acids with at least 20 carbon atoms and more than 3 double bonds.

Table 3. Effect of nutrient deficiencies on growth performance of gilthead sea bream fed to visual satiety from May to July (13 weeks). Data on body weight, feed intake, growth indices and body composition are the mean (SEM) of triplicate tanks. Data on viscera, mesenteric fat and liver weight are the mean (SEM) of 20 fish. Different superscript letters in each row indicate significant differences among dietary treatments (SNK test, P<0.05).

	Diet							
	CTRL	SAA	n-3 LC-PUFA	PL	P	Min	Vit	P-value[1]
Initial body weight (g)	15.1(0.06)	15.1(0.21)	15.2(0.14)	15.0(0.25)	15.1(0.11)	15.0(0.04)	15.2(0.08)	0.977
Final body weight (g)	85.5(1.58)^{a}	72.5(1.41)^{b}	51.2(0.17)^{d}	71.7(1.80)^{b}	60.2(0.66)^{c}	71.5(2.12)^{b}	66.1(0.65)^{bc}	*<0.001*
Feed intake (g DM/fish)	65.9(1.46)^{a}	54.7(1.36)^{b}	37.9(0.68)^{d}	49.6(2.16)^{bc}	51.4(0.91)^{bc}	51.6(1.73)^{bc}	46.9(0.59)^{c}	*<0.001*
Viscera (g)	6.39(0.27)^{a}	5.34(0.23)^{b}	4.40(0.21)^{d}	5.80(0.19)^{ab}	5.79(0.18)^{b}	5.54(0.20)^{abc}	4.90(0.16)^{cd}	*<0.001*
Mesenteric fat (g)	1.14(0.13)^{a}	0.92(0.12)^{abc}	0.58(0.09)^{c}	0.79(0.08)^{bc}	1.27(0.11)^{a}	1.04(0.11)^{ab}	0.49(0.06)^{c}	*<0.001*
Liver (g)	1.09(0.06)^{a}	0.80(0.03)^{c}	0.98(0.04)^{ab}	1.01(0.05)^{ab}	0.87(0.02)^{bc}	0.77(0.02)^{c}	0.79(0.03) ^{c}	*<0.001*
VSI (%)[2]	7.12(0.20)^{bc}	7.03(0.22)^{bc}	7.81(0.20)^{b}	7.43(0.16)^{bc}	8.95(0.23)^{a}	7.10(0.19)^{bc}	6.92(0.16)^{c}	*<0.001*
MSI (%)[3]	1.27(0.13)^{b}	1.19(0.14)^{bc}	0.98(0.12)^{bc}	0.99(0.10)^{bc}	1.97(0.16)^{a}	1.32(0.13)^{b}	0.70(0.08)^{c}	*<0.001*
HSI (%)[4]	1.23(0.04)^{bc}	1.05(0.02)^{cd}	1.76(0.08)^{a}	1.29(0.04)^{b}	1.36(0.04)^{b}	0.99(0.02)^{d}	1.10(0.04)^{cd}	*<0.001*
SGR (%)[5]	1.97(0.02)^{a}	1.82(0.04)^{b}	1.38(0.01)^{d}	1.77(0.05)^{b}	1.57(0.01)^{c}	1.77(0.03)^{b}	1.67(0.01)^{b}	*<0.001*
FE (%)[6]	1.07(0.01)^{a}	1.08(0.01)^{a}	0.95(0.02)^{b}	1.10(0.01)^{a}	0.88(0.01)^{c}	1.09(0.01)^{a}	1.08(0.01)^{a}	*< 0.001*
Whole body composition (% wet weight)								
Moisture	67.6(0.21)^{b}	68.3(0.07)^{b}	69.9(0.31)^{a}	69.70(0.07)^{a}	66.3(0.08)^{c}	68.2(0.27)^{b}	70.3(0.34)^{a}	*<0.001*
Crude protein	17.5(0.16)^{a}	16.4(0.29)^{ab}	16.5(0.3)^{ab}	16.5(0.25)^{ab}	15.8(0.08)^{b}	17.5(0.17)^{ab}	16.9(0.38)^{ab}	*0.017*
Crude lipid	9.28(0.32)^{a}	8.71(0.10)^{ab}	8.03(0.13)^{ab}	8.25(0.51)^{ab}	8.41(0.52)^{ab}	9.03(0.24)^{b}	6.95(0.08)^{b}	*0.005*
Retention, % intake								
Nitrogen	37.0(0.63)^{a}	33.4(0.49)^{ab}	31.7(0.46)^{bc}	34.5(0.31)^{ab}	27.7(0.45)^{c}	36.0(0.45)^{a}	36.1(1.33)^{a}	*<0.001*
Lipid	64.3(1.19)^{a}	58.8(0.92)^{b}	46.9(0.73)^{c}	57.4(0.58)^{b}	50.1(0.84)^{c}	65.6(1.29)^{a}	46.6(1.98)^{c}	*<0.001*

[1]Result values from one-way analysis of variance; [2]Viscerosomatix index = (100 x viscera weight) / fish weight
[3]Mesenteric index = (100 x mesenteric fat weight) / fish weight; [4]Hepatosomatic index = (100 x liver weight) / fish weight
[5]Specific growth rate = 100 x (ln final body weight - ln initial body weight) / days; [6]Feed Efficiency = wet weight gain / dry feed intake

Table 4. Effect of nutrient deficiencies on basic plasma biochemistry of sea bream fed to visual satiety from May to July (13 weeks). Data are the mean (SEM) of 10 fish. Different superscript letters in each row indicate significant differences among dietary treatments (SNK test, P<0.05).

	CTRL	SAA	n-3 LC-PUFA	PL	P	Min	Vit	P-value[1]
Haemoglobin (g/dL)	7.16(0.47)^{ab}	6.62(0.30)^{ab}	5.90(0.36)^{b}	7.09(0.42)^{ab}	7.79(0.47)^{a}	7.19(0.34)^{ab}	8.13(0.47)^{a}	0.008
Haematocrit (%)	32.8(1.68)^{a}	34.1(1.33)^{a}	27.4(0.88)^{b}	30.5(0.97)^{ab}	34.9(1.69)^{a}	36.2(1.63)^{a}	32.8(1.48)^{a}	0.001
RBC x 10^{-6}/mL	2.66(0.07)^{a}	2.53(0.09)^{a}	2.07(0.05)^{b}	2.31(0.13)^{b}	2.53(0.11)^{ab}	2.80(0.08)^{a}	2.79(0.09)^{a}	<0.001
MCF (g/L)	6.62(0.21)^{a}	nd	7.37(0.24)^{b}	6.90(0.14)^{ab}	nd	nd	nd	0.048
Glucose (mg/dl)	56.1(2.86)	58.3(2.86)	56.0(2.13)	52.3(1.40)	57.5(3.10)	57.3(1.83)	50.6(1.96)	0.099
Triglycerides (mM)	0.87(0.09)^{a}	0.94(0.10)^{a}	0.53(0.05)^{b}	0.86(0.09)^{a}	2.22(0.26)^{c}	0.86(0.06)^{a}	0.62(0.08)^{ab}	<0.017
Total cholesterol (mg/dL)	152.2(10.7)^{a}	130.5(3.91)^{ab}	65.62(7.36)^{c}	113.6(5.52)^{b}	192.5(9.67)^{d}	139.9(9.66)^{a}	90.7(5.23)^{e}	<0.001
HDL cholesterol (mg/dL)	119.0(9.38)^{a}	123.6(5.68)^{a}	64.2(3.76)^{b}	110.7(6.39)^{ad}	159.7(8.44)^{c}	120.1(11.9)^{a}	89.9(6.96)^{d}	<0.001
VLDL/LDL cholesterol (mg/dL)	34.1(9.31)^{ab}	18.9(1.58)^{ab}	16.5(1.39)^{a}	22.8(3.33)^{ab}	52.2(6.38)^{b}	23.2(2.16)^{ab}	18.4(2.62)^{a}	0.012
Total proteins (g/L)	33.8(1.81)^{a}	28.4(0.66)^{b}	24.1(2.05)^{b}	26.3(1.37)^{b}	38.1(1.85)^{a}	35.3(1.86)^{a}	26.1(2.23)^{b}	<0.001
ALAT (U/L)	3.21(0.46)	3.15(0.24)	2.29(0.29)	2.83(0.35)	2.78(0.37)	2.56(0.27)	2.36(0.41)	0.362
ASAT (U/L)	19.5(1.31)	19.9(1.86)	15.1(4.03)	24.1(7.62)	16.5(2.43)	25.6(5.96)	17.1(1.86)	0.507
GLDH (U/L)	2.25(0.17)	1.95(0.13)	2.02(0.25)	1.63(0.29)	1.43(0.30)	1.59(0.24)	1.18(0.32)	0.078
ALP (U/L)	78.8(6.34)^{a}	80.6(4.12)^{a}	79.2(5.41)^{a}	60.8(2.41)^{b}	101.1(8.52)^{c}	92.6(6.33)^{c}	76.2(4.65)^{ac}	0.002
Creatinine (mg/dL)	0.12(0.01)^{a}	0.10(0.01)^{a}	0.06(0.01)^{b}	0.10(0.01)^{a}	0.12(0.01)^{a}	0.10(0.01)^{a}	0.05(0.01)^{b}	<0.001
Choline (µM)	15.1(1.30)^{a}	10.6(0.6)^{bc}	15.5(1.61)^{a}	13.2(1.11)^{ab}	13.8(0.97)^{ab}	10.4(0.77)^{bc}	9.00(0.57)^{c}	<0.001
Calcium (mg/dL)	11.2(0.50)^{a}	11.2(0.26)^{a}	9.78(0.52)^{a}	10.4(0.28)^{a}	11.8(0.76)^{a}	10.9(0.32)^{a}	6.64(1.64)^{b}	<0.001
Chloride (mg/dL)	448.6(6.60)	445.0(7.75)	472.6(5.82)	464.9(10.9)	457.4(12.5)	451.2(5.22)	451.2(12.9)	0.370
Magnesium (mg/mL)	1.82(0.17)	1.81(0.05)	1.67(0.04)	1.71(0.09)	1.75(0.21)	1.89(0.10)	1.78(0.14)	0.923
Phosphate (mg/dL)	14.7(0.40)^{a}	13.1(0.45)^{a}	14.01(0.52)^{a}	13.7(0.0.45)^{a}	7.94(0.25)^{b}	12.9(0.32)^{a}	14.8(0.55)^{b}	<0.001
Antioxidant capacity (Trolox mM)	0.25(0.03)^{a}	0.37(0.03)^{b}	0.41(0.03)^{b}	0.43(0.03)^{b}	0.61(0.03)^{c}	0.65(0.06)^{c}	0.68(0.03)^{c}	<0.001
Lysozyme (U/L)	97.7(25.98)	14.4(5.01)	70.7(35.90)	98.7(16.56)	51.5(18.30)	35.5(14.37)	68.0(18.40)	0.122
Respiratory burst (IRLU)	337.0(54.6)^{a}	336.5(37.7)^{a}	1933.5(254.9)^{b}	479.0(104.1)^{a}	332.50(39.2)^{a}	326.0(32.3)^{a}	365.0(37.7)^{a}	<0.001
GH (ng/mL)	5.13(48)^{a}	7.35(0.94)^{ab}	14.0(0.23)^{c}	10.0(2.04)^{b}	13.3(2.16)^{c}	10.1(1.31)^{b}	12.3(0.97)^{c}	<0.001
IGF-I (ng/mL)	96.7(0.85)^{a}	78.7(5.15)^{b}	45.1(4.34)^{c}	69.1(4.68)^{b}	53.1(3.88)^{c}	66.6(2.61)^{b}	64.6(4.61)^{b}	<0.001

[1]Result values from one-way analysis of variance. nd, not determined

Table 5. Summary of the histological features observed in the liver and anterior (AI) and posterior (PI) intestine of fish fed control (CTRL) and nutrient deficient diets. The intensity of the features was graded from absence (-) to the highest observed level (+++).

Tissue	Trait	CTRL	SAA	n-3 LC-PUFA	PL	P	Min	Vit
Liver	Lipid droplets	-	+	++	+/-	+	+	-
	Glycogen PAS+	-	++	+	-	++	+	++
AI	Goblet cells PAS+	++	++	-	-	-	-	+
	Lipid droplets	-	-	+++	++	-	-	++
	Submucosal granulocytes	-	-	-	+	-	+	++
	Lymphocytes	-	-	-	-	-	-	+
PI	Goblet cells PAS+	+	-	-	+	-	-	+
	Goblet cells Giemsa+	++	++	+	++	-	-	-
	Submucosal granulocytes	-	-	-	-	-	+	+
	Epithelial lymphocytes	-	-	-	+	-	-	+
	Rodlet cells	-	-	-	+	+	++	+

Figure 1

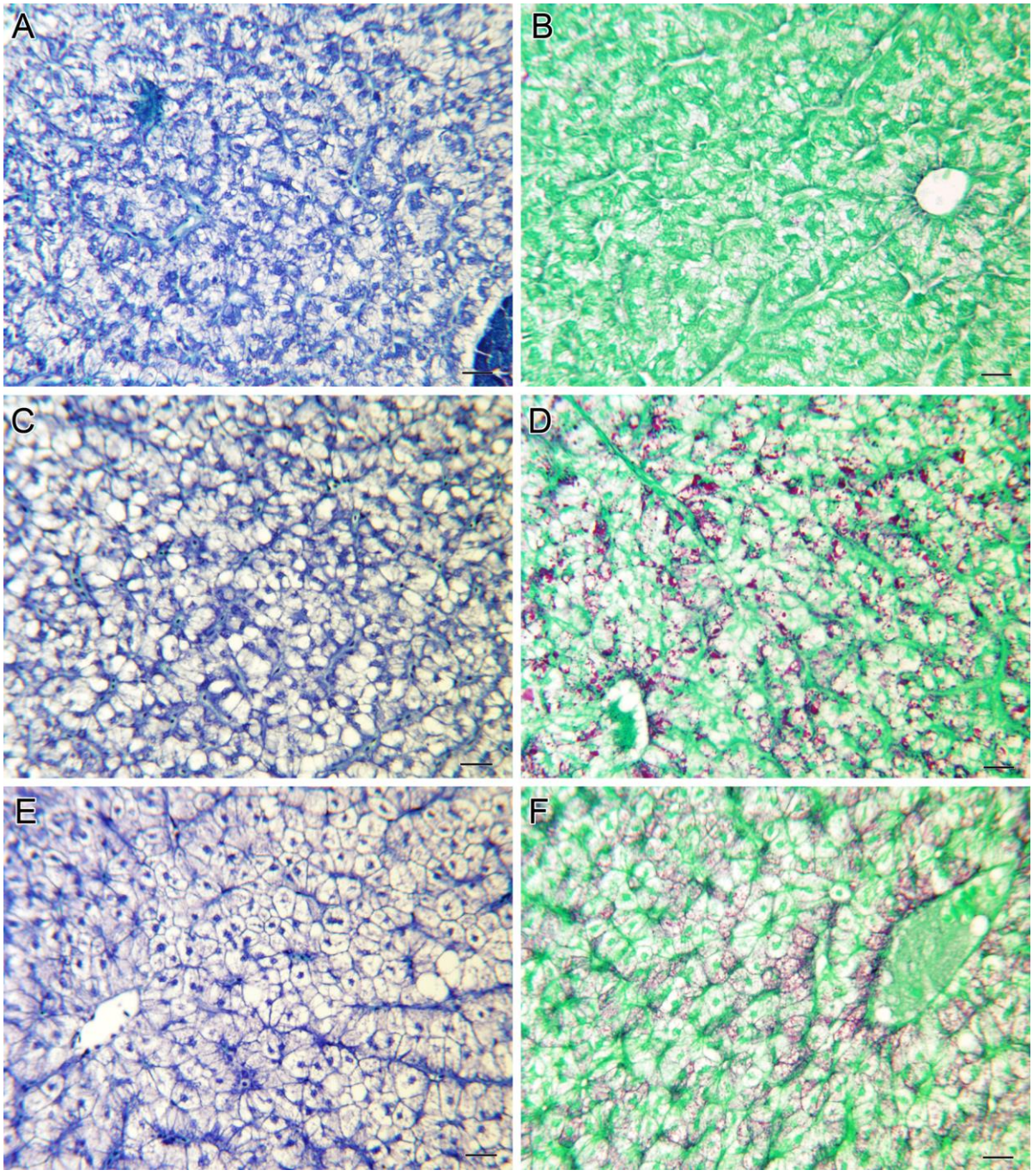


Figure 2

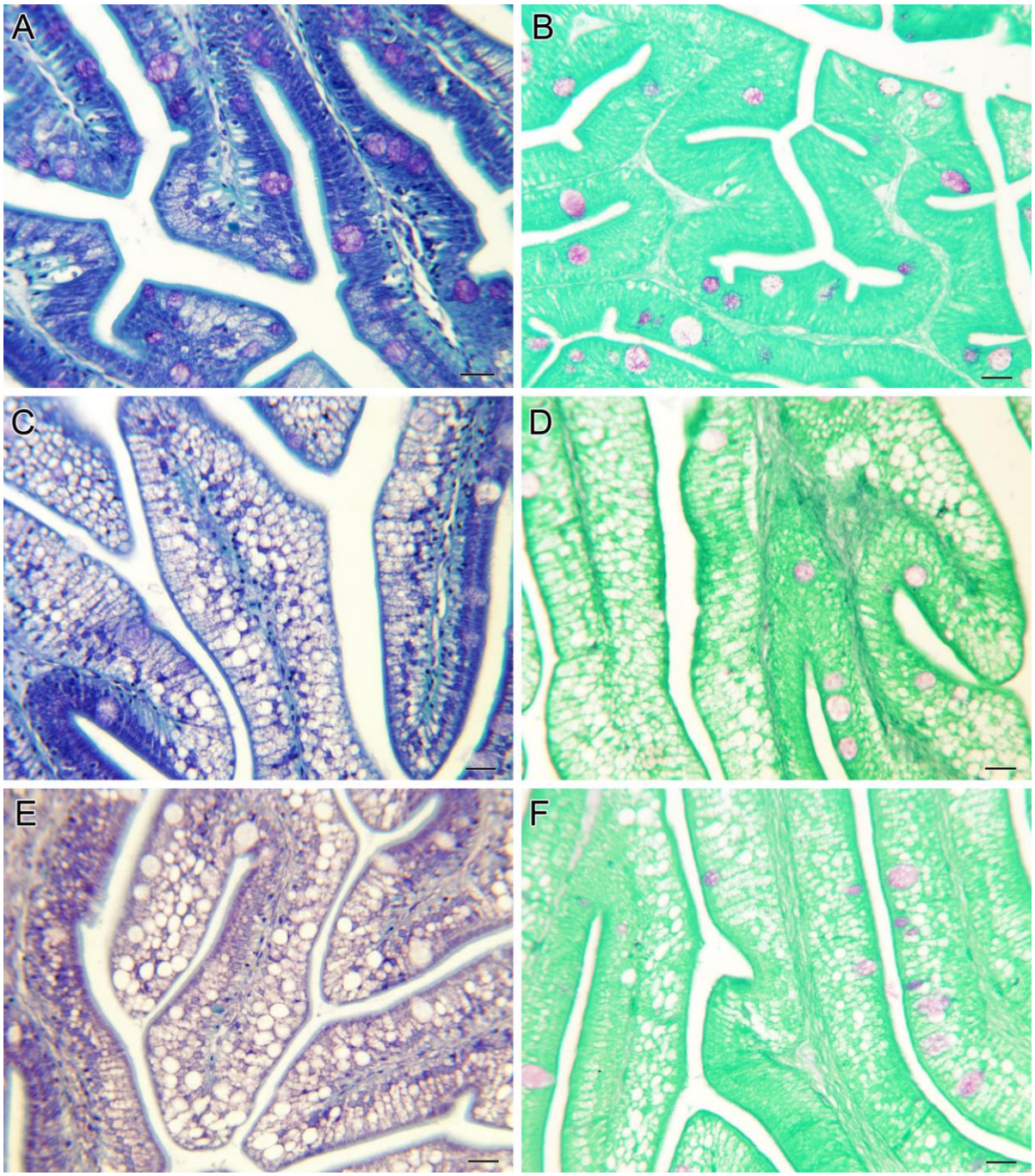
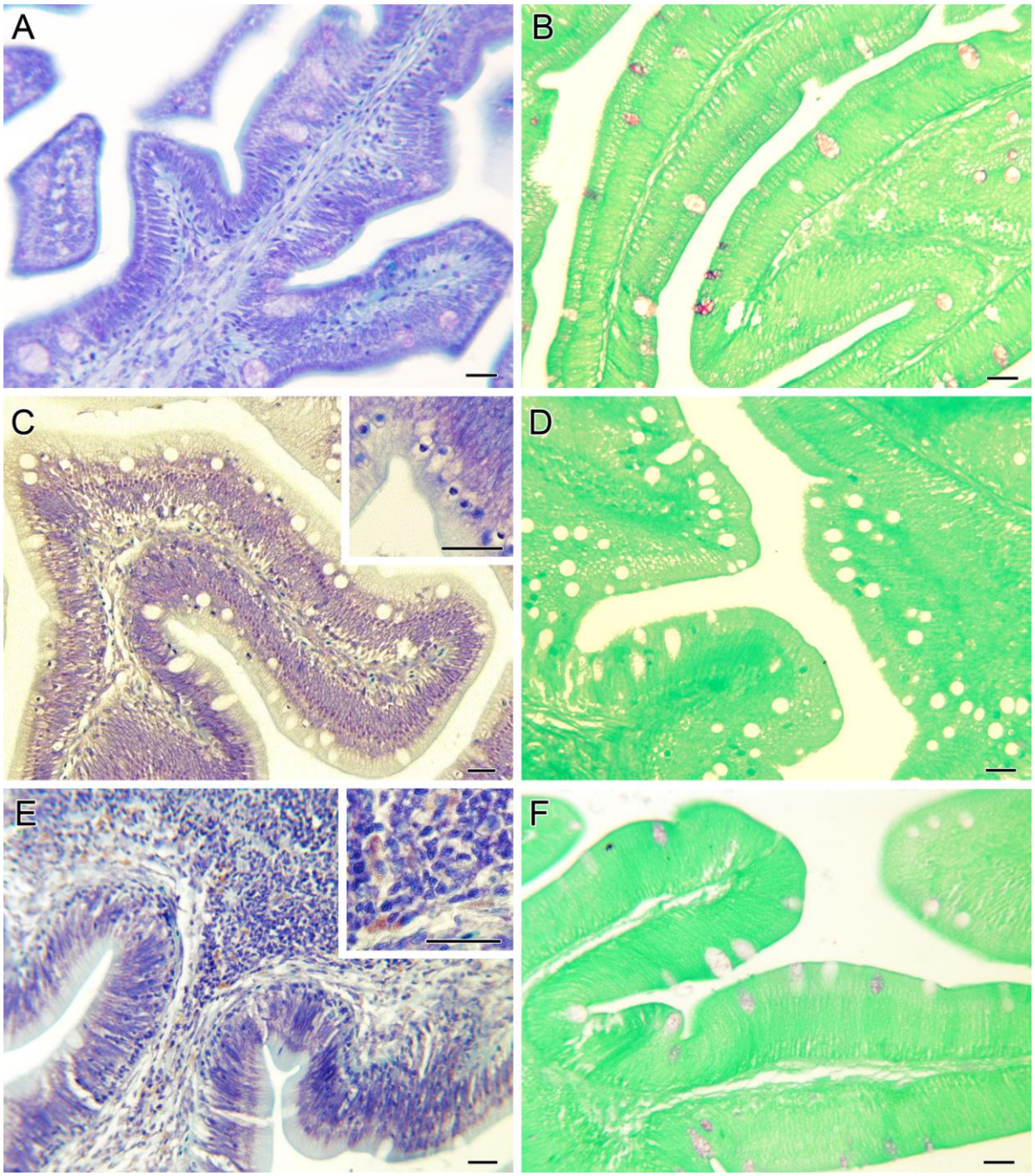


Figure 3



Supplemental Table 1. Set of clinical signs for the diagnosis of nutrient deficiencies in gilthead sea bream fed nutrient deficient diets.*, indicates the magnitude of change.

PARAMETER CATEGORY/ANALYTE		DIET					
		SAA	n-3 LC-PUFA	PL	P	Min	Vit
Growth performance	Growth	Decreased	Decreased*	Decreased	Decreased	Decreased	Decreased
	Feed efficiency	No change	Decreased	No change	Decreased*	No change	No change
	Feed intake	Decreased	Decreased*	Decreased	Decreased	Decreased	Decreased
Somatic indexes	Hepatosomatic index	No change	Increased	No change	No change	Decreased	No change
	Mesenteric fat index	No change	Decreased	Decreased	Increased	No change	Decreased*
	Viscerosomatic index	No change	No change	No change	Increased	No change	No change
Nutrient retention	Fat retention	Decreased	Decreased*	Decreased	Decreased	No change	Decreased*
	Nitrogen retention	No change	Decreased	No change	Decreased*	No change	No change
Haematology	Hematocrit	No change	Decreased	No change	No change	No change	No change
	Hemoglobin	No change	Decreased	No change	No change	No change	No change
	Red Blood Cells	No change	Decreased	No change	No change	No change	No change
Blood biochem/electrolytes	Calcium	No change	No change	No change	No change	No change	Decreased
	Chloride	No change	No change	No change	No change	No change	No change
	Magnesium	No change	No change	No change	No change	No change	No change
	Phosphate	No change	No change	No change	Decreased	No change	No change
Blood biochem/metabolites	Glucose	No change	No change	No change	No change	No change	No change
	Triglycerides	No change	Decreased	No change	Increased	No change	Decreased
	Total cholesterol	No change	Decreased*	No change	Increased	No change	Decreased
	Total proteins	Decreased	Decreased	Decreased	No change	No change	Decreased
	Creatinine	No change	Decreased	No change	No change	No change	Decreased
	Choline	Decreased	No change	No change	No change	Decreased	Decreased*
Blood biochem/enzymes	ALAT	No change	No change	No change	No change	No change	No change
	ASAT	No change	No change	No change	No change	No change	No change
	GLDH	No change	No change	No change	No change	No change	No change
	ALP	No change	No change	Decreased	Increased	Decreased	Decreased
Blood biochem/	Antioxidant capacity	Increased	Increased	Increased	Increased*	Increased*	Increased*
oxidative/inflammatory condition	Respiratory burst	No change	Increased	No change	No change	No change	No change
	Lysozyme	No change	No change	No change	No change	No change	No change
Blood biochem/hormones	Growth Hormone	Increased	Increased	Increased*	Increased	Increased	Increased
	IGF-I	Decreased	Decreased	Decreased*	Decreased	Decreased	Decreased
Histology/liver	Glycogen depots	Increased	Increased	No change	Increased	Increased	No change
	Lipid droplets	Increased	Increased*	No change	Increased	Increased	No change
Histology/ant. intestine	Lipid droplets	No change	Increased*	Increased	No change	No change	Increased
	Lymphocyte submucosa	No change	No change	No change	No change	No change	Increased
	Granulocyte submucosa	No change	No change	Increased	No change	Increased	Increased*
	GC PAS +	No change	Decreased	Decreased	Decreased	Decreased	Decreased
Histology/post. intestine	Lipid droplets	No change	No change	No change	No change	No change	No change
	Lymphocyte submucosa	No change	No change	Increased	No change	No change	Increased
	Granulocyte submucosa	No change	No change	No change	No change	Increased	Increased
	GC PAS +	No change	No change	Increased	No change	No change	Increased
	GC Giemsa +	No change	Decreased	No change	Decreased	Decreased	Decreased
	Rodlet cells	No change	No change	Increased	Increased	Increased*	Increased

Supplemental Table 2. Reference values for organosomatic indexes and basic blood biochemistry in farmed gilthead sea bream.

ORGANOSOMATIC INDEXES	Environmental condition	Fish condition	Mean	Lower limit	Upper limit	Unit	Comments
Hepatosomatic index (HSI)	Summer	Fast growth	1.5	1	2	%	HSI is lowered by deficiencies in minerals and vitamins. Liver fat deposition and HSI are increased by deficiencies in EFA, and liver steatosis is exacerbated in winter.
	Late autumn	Slow growth	2.1	1.6	3	%	
Mesenteric index (MSI)	Summer	Fast growth	1.7	1	2.5	%	MSI is increased by P deficiencies. MSI is lowered by deficiencies in SAA, EFA, PL and vitamins.
	Late autumn	Slow growth	1.2	0.7	2	%	
HAEMATOLOGY	**Environmental condition**	**Fish condition**	**Mean**	**Lower limit**	**Upper limit**	**Unit**	**Comments**
Haemoglobin (Hb)	Summer	Fast growth	5.7	4	8	g/dl	Hb concentration is decreased by EFA deficiencies and low FM diets. High Hb concentrations > 12 g/dl are found in hypoxic conditions (oxygen saturation < 20%).
	Late autumn	Slow growth	6.5	5	9	g/dl	
Haematocrit (Hct)	Summer	Fast growth	35.5	25	45	%	Low Hc values are found in fish with clinical signs of EFA deficiencies. Hc values > 50% are found in hypoxic conditions (oxygen saturation < 20%).
	Late autumn	Slow growth	32.5	20	40	%	
Red blood cells (RBC)	Summer	Fast growth	2.4	2	3	10^{-6}/ml	RBC counts (< 2 million/ml) are low in fish with clinical signs of EFA deficiencies. This is a type of haemolytic anaemia with an enhanced erythroid osmotic fragility
	Late autumn	Slow growth	2.9	2.5	3.5	10^{-6}/ml	

							(MCF>7 g/l).
ELECTROLYTES	**Environmental condition**	**Fish condition**	**Mean**	**Lower limit**	**Upper limit**	**Unit**	**Comments**
Chloride (Cl)	Summer/late autumn	Fast/slow growth	450	350	500	mg/dl	Poorly regulated at the nutritional level.
Calcium (Ca)	Summer	Fast growth	13.4	12	16	mg/dl	Hypocalcaemia (< 7 mg/dl) is found in fish with clinical signs of vitamin deficiencies.
	Late autumn	Slow growth	12.5	10	14	mg/dl	
Phosphate (HPO_4^{2-})	Summer/late autumn	Fast/slow growth	11.4	9.5	14	mg/dl	Low phosphate concentrations (< 9 mg/dl) are found in fish with clinical signs of P deficiencies.
Magnesium (Mg)	Summer/late autumn	Fast/slow growth	1.9	1.5	2.2	mg/ml	Poorly regulated at the nutritional level.
METABOLITES	**Environmental condition**	**Fish condition**	**Mean**	**Lower limit**	**Upper limit**	**Unit**	**Comments**
Glucose	Summer/late autumn	Fast/slow growth	52.6	45	90	mg/dl	Basal glucose levels are poorly influenced by fish size. Consistent hyperglycaemia (> 125 mg/dl) is found in hypoxic conditions and crowding stress.
Lactate	Spring/early summer	Fast growth	9	8	11	mg/dl	High plasma lactate concentrations (> 12 mg/dl) are found in hypoxic steady states.
Triglycerides	Summer	Fast growth	0.59	0.5	1	mM	Summer hypertriglyceridemia (> 1.5 mM) is found in fish with clinical signs of P deficiencies.
	Late autumn	Slow growth	2.7	0.7	4	mM	
Total cholesterol	Summer	Fast growth	140	100	200	mg/dl	Summer hypocholesterolemia (< 100 mg/dl) is found in fish with clinical

	Late autumn	Slow growth	210	150	300	mg/dl	signs of deficiencies in EFA, PL and vitamins. Summer hypercholesterolemia (> 170 mg/dl) is found in fish with clinical signs of P deficiencies.
METABOLITES	**Environmental condition**	**Fish condition**	**Mean**	**Lower limit**	**Upper limit**	**Unit**	**Comments**
Proteins	Summer	Fast growth	41	35	45	mg/dl	Summer hypoproteinemia (< 30 mg/ml) is found in fish with clinical signs of deficiencies in SAA, EFA, PLs and vitamins.
	Late autumn	Slow growth	50	45	55	mg/dl	
Creatinine	Summer	Fast growth	0.12	0.08	0.15	mg/dl	Reduced creatinine levels (< 0.07 mg/dl) are found in summer growing fish with clinical signs of deficiencies in vitamins and EFA.
	Late autumn	Slow growth	0.20	0.1	0.3	mg/dl	
Choline	Summer	Fast growth	16	11.5	22	µM	Reduced choline levels (< 10.5 ng/ml) are found in summer growing fish with clinical signs of deficiencies in SAA, minerals and vitamins.
	Late autumn	Slow growth	7.2	5	10	µM	
ENZYMES	**Environmental condition**	**Fish condition**	**Mean**	**Lower limit**	**Upper limit**	**Unit**	**Comments**
Alanine aminotransferase (ALAT)	Summer/late autumn	Fast/slow growth	1.4	0.5	3	U/l	No clinical histopathological signs are found with this range of variation.
Aspartate aminotransferase (ASAT)	Summer/late autumn	Fast/slow growth	15	5	40	U/l	No clinical histopathological signs are found with this range of variation.
Glutamate dehydrogenase (GLDH)	Summer/late autumn	Fast/slow growth	0.7	0.2	1.5	U/l	No clinical histopathological signs are found with this range of

							variation.
Alkaline phosphatase (ALP)	Summer	Fast growth	98	75	125	U/l	Plasma ALP activity is increased by P deficiencies. Low ALP activities are found in summer growing fish with clinical signs of PL and vitamin deficiencies (< 70 U/l)
	Late autumn	Slow growth	85	55	100	U/l	

INFLAMATORY & ANTIOXIDANT STATUS	Environmental condition	Fish condition	Mean	Lower limit	Upper limit	Unit	Comments
Lysozyme	Summer	Fast growth	150	50	300	U/l	Reduced levels in fish with clinical signs of deficiencies in SAA.
	Late autumn	Slow growth	55	20	100	U/l	
Respiratory burst	Summer	Fast growth	500	300	600	IRLU	Marked increases (more than 4-5 fold) in fish with deficiencies in EFA.
	Late autumn	Slow growth	4000	500	10000	IRLU	
Total antioxidant capacity (TAC, Trolox mM)	Summer	Fast growth	0.9	0.5	1	Trolox (mM)	TAC is highly influenced by the environment and nutritional condition, and positively correlates for a given physiological conditions with the risk of oxidative stress. Increased values for TAC are reported in fish with clinical signs of deficiencies in vitamins, minerals and P. Increased TAC is also reported in hypoxia.
	Late autumn	Slow growth	1.1	0.8	1.25	Trolox (mM)	
HORMONES	**Environmental condition**	**Fish condition**	**Mean**	**Lower limit**	**Upper limit**	**Unit**	**Comments**
Growth hormone (GH)	Summer	Fast growth	7.5	3	10	ng/ml	Circulating levels of GH are nutritionally and seasonality regulated. Malnutrition induces a state of GH resistance and marked increases in circulating GH concentrations (> 10 ng/ml) are found in fish with clinical signs of specific nutrient deficiencies and growth impairment.
	Late autumn	Slow growth	2.5	1.5	3	ng/ml	

Insulin-like growth factor-I (IGF-I)	Summer	Fast growth	85	50	110	ng/ml	Low IGF-I concentrations in combination with high GH concentrations are strong markers of malnutrition and impaired growth.